

Europäisches Patentamt  
European Patent Office  
Office européen des brevets



(11) **EP 1 265 073 A2**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:  
11.12.2002 Bulletin 2002/50

(51) Int Cl.7: **G01N 33/94, G01N 33/569,  
G01N 33/68**

(21) Application number: **02077275.2**

(22) Date of filing: **05.06.2002**

(84) Designated Contracting States:  
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU  
MC NL PT SE TR**  
Designated Extension States:  
**AL LT LV MK RO SI**

(72) Inventors:  
• **Xie, Dong**  
**Germantown, Midland 20874 (US)**  
• **Cao, Wei**  
**Centereach, New York 11720 (US)**  
• **Erickson, John W.**  
**Frederick, Midland 21703 (US)**

(30) Priority: **05.06.2001 US 295557 P**

(71) Applicant: **Tibotec Pharmaceuticals Ltd.**  
**Blanchardstown, Dublin 15 (IE)**

(74) Representative: **Daelemans, Frank F.R. et al**  
**Tibotec-Virco N.V.,**  
**General De Wittelaan L11 B3**  
**B-2800 Mechelen (BE)**

(54) **Methods for determining plasma free drug concentration**

(57) The present invention relates to methods for isothermal titration calorimetry analysis of the binding affinity of protease inhibitors to plasma proteins.

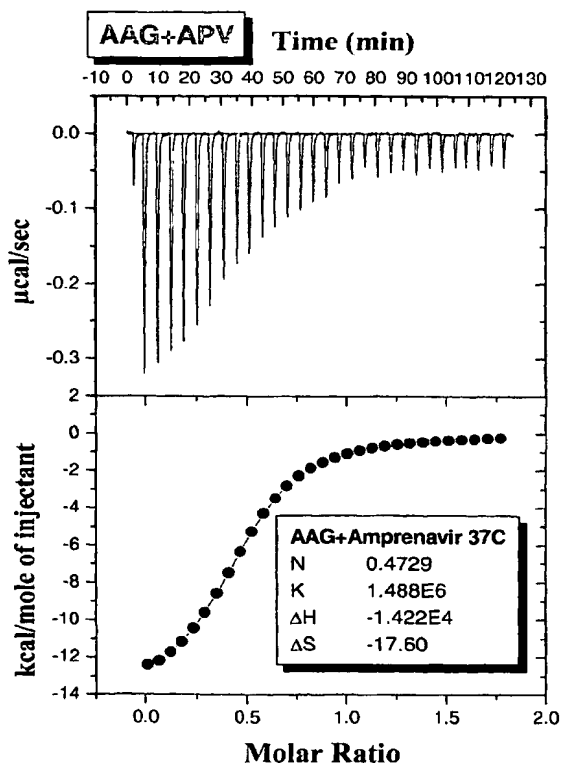


Figure 1

**Description****Field of the Invention**

- 5 **[0001]** The present invention relates to methods for isothermal titration calorimetry analysis of the binding affinity of protease inhibitors to plasma proteins.

**Background of the invention**

- 10 **[0002]** Following administration, drugs are transported in biological fluids (e.g. in blood) partly in solution as free drug and partly bound to blood components (e.g., plasma proteins, blood cells). The physiologically active substances are in equilibrium between a free form and a form bound to endogenous ligands present in the same fluids (see reviews by Kremer, et al. Pharmacol Rev. 1988, 40:1-47). Only free drug is available for passive diffusion to target tissue sites where the desired biological activity may take place. When compared to the total-substance level, the free drug concentration is more closely related to drug concentration at the active site, to drug effects, and to clinical effectiveness. Observations made on both healthy and pathologically afflicted humans confirmed that the patients' clinical condition correlates better with variations in free form concentration when compared to variations in total substance concentrations.

- 20 **[0003]** Slight changes in the binding affinity of drugs to blood components can result in significant changes in clinical response or even cause a toxic response. Since it is the free drug in plasma which equilibrates with the pharmacologically active site, a slight change in the binding affinity, such as from 99 to 98 % binding, can result in an almost 100 % change in free drug concentration, and, thus, can cause a significant alteration in response. This is the case for most HIV protease inhibitors (PIs) (Acosta, Acquir Immune Defic Syndr. 2002 Feb 1; 29 Suppl 1:S11-8; Sadler, et al. Antimicrob Agents Chemother. 2001 Mar; 45(3):852-6; Anderson, et al, AIDS. 2000 Oct 20;14(15):2293-7; Bilello, et al., J Infect Dis. 1995 Mar;171(3):546-51).

- 25 **[0004]** The binding of drugs to plasma proteins may influence their distribution, elimination and pharmacological effect, which is considered more closely related to unbound rather than total drug concentration (du Souich et al, 1993, Clin Pharmacokinet. 24:435-40.). Human serum albumin (HSA) and human  $\alpha_1$ -acid glycoprotein (AAG) are two mainly involved proteins in the binding of HIV PIs in plasma. Human AAG is an acute-phase protein whose expression increases during acute inflammatory episodes, infections, injuries, neoplastic disease, and AIDS (Kremer et al, Pharmacol Rev. 1988, 40: 1-47; Oie et al, 1993, J Acquir Immune Defic Syndr Hum Retrovirol. 5:531-533; Mackiewicz et al, 1995, Glycoconj. J. 12:241-247; van Dijk et al, 1995, Glycoconj J. 12:227-233). The level of AAG in human serum fluctuates between 0.15 and 1.5 mg/mL, and the average value may vary by as much as 4-fold between healthy volunteers and AIDS patients (Kremer et al, Pharmacol Rev. 1988, 40: 1-47; Oie et al, 1993, J Acquir Immune Defic Syndr Hum Retrovirol. 5:531-533). Additionally, AAG concentrations have been suggested to vary by race or ethnicity (Johnson et al, 1997, J. Pharm. Sci. 86: 1328-1333). It has been reported that AAG exists as a mixture of two or three genetic variants (the A variant and the F1 and/or S variants) in the plasma of most individuals (Hervé et al, 1998, Mol. Pharmacol. 54:129-138), which present different drug binding specificities.

- 30 The question whether AAG binding had an effect on *in vivo* antiviral activity of anti-HIV PIs has recently heightened the great interest in the investigations of effects of serum proteins on activity and pharmacokinetics of anti-HIV PIs *in vitro* (Bilello et al. 1995, J. Infect. Dis. 171:546-551; Bilello et al. 1996, Antimicrobiol. Agents Chemother. 40:1491-1497; Lazdins et al. 1996, J. Infect. Dis. 175:1063-1070; Kiriya et al. 1996, Biopharmac. Drug Dispos. 17:739-751; Zhang et al. 1999, J. Infect. Dis. 180:1833-1837; Jones et al. 2001, Br J Clin Pharmacol. 51:99-102; Kageyama et al. 1994, Antimicrob Agents Chemother. 22:499-506; Livingstone et al. 1995, J. Infect Dis. 172:1238-45), and *in vivo* (Sadler et al. 2001, Antimicrob. Agents Chemother. 45:852-856.). *In vitro* these studies have consistently demonstrated that human AAG reduced the antiviral activity of most PIs by decreasing the amount of free drug available for interaction with the drug target. Studies *in vitro* by Bilello et al. (1995, J. Infect. Dis. 171:546-551; 1996, Antimicrobiol. Agents Chemother. 40:1491-1497) have shown that the antiviral efficacy of two HIV PIs, A77003 and A80978, decreased as the concentration of AAG was increased and that the inhibition of HIV protease was highly correlated with the amount of intracellular inhibitor. Also, the clinical significance of these effects *in vitro* was shown by the lack of clinical efficacy of the HIV PI SC-52151, which has potent antiretroviral activity *in vitro* but inactivity *in vivo*, because extensive protein binding prevented intracellular diffusion (Fischl et al. J Acquir Immune Defic Syndr Hum Retrovirol 1997, 15:28-34). While there are extensive data to address this problem, no general correlation between protein binding and anti-HIV activity can be made so far on the basis of these studies.

- 35 Although the precise site of action of PIs has not been defined, the inhibition of HIV protease is possibly to take place intracellularly. Bilello et al. (Bilello et al, 1996, Antimicrobiol. Agents Chemother. 40:1491-1497) have demonstrated that cellular uptake of protease inhibitor is proportionally decreased in the presence of AAG, which results in a decreased antiviral activity. These observations indicate that not only the antiviral EC<sub>50</sub> (50% effective concentration) of

PIs, but also their interaction with human AAG probably are the most important determinant factors of their anti-HIV efficacy *in vivo* because only free drug in plasma can equilibrate with intracellular compartments.

[0005] In determining therapeutic amounts and subsequent dosage regimens for individual protease inhibitors, it is clinically relevant to establish the binding affinities of the different protease inhibitors to plasma proteins. Also of interest is knowledge about plasma protein binding sites and the free drug concentrations. These factors and the information extracted therefrom will aid into obtaining a more complete pharmacokinetic profiling of protease inhibitors, which will result in more accurate and effective therapeutic amounts and dosage regimens for the protease inhibitors, which will ultimately translate in an improved treatment for HIV infected patients. To date, binding of a particular protease inhibitor to plasma proteins is expressed as a percentage of total amount of drug that is bound to the plasma proteins Sadler, et al. *Antimicrob Agents Chemother.* 2001 Mar;45(3):852-6; Anderson, et al. *AIDS.* 2000 Oct 20;14(15):2293-7; Bilello, et al., *J Infect Dis.* 1995 Mar;171(3):546-51. This is an absolute number for a particular concentration of drug and plasma protein. It is well established that the concentration of plasma proteins, in particular the two most important ones in HIV/AIDS therapy AAG and serum albumin (HSA), is variable depending on age, race, and disease state (Kremer, et al. *Pharmacol Rev.* 1988 Mar;40(1):1-47). Furthermore, total drug concentration is also different in individuals due to their various rate of absorption, distribution, metabolism, and excretion. Therefore, the use of percentage of bound and free forms is not useful and cannot be generally applied. However, if the dissociation constant,  $K_d$ , is directly determined for the binding of drug to the plasma protein, and the total drug and plasma concentration is known from pharmacokinetic studies, the concentrations of the free and bound form of drug can be readily calculated (Wyman, J. and Gill, S., "Binding and Linkage", 1990, Published by University Science Books, Mill Valley, California).

[0006] Although a considerable literature regarding the effect of AAG-binding on antiviral PIs has arisen (Bilello et al. 1995, *J. Infect. Dis.* 171:546-551; Bilello et al. 1996, *Antimicrobiol. Agents Chemother.* 40:1491-1497; Lazdins et al. 1996, *J. Infect. Dis.* 175:1063-1070; Kiriya et al. 1996, *Biopharmac. Drug Dispos.* 17:739-751; Zhang et al. 1999, *J. Infect. Dis.* 180:1833-1837; Jones et al. 2001, *Br J Clin Pharmacol.* 51:99-102; Kageyama et al. 1994, *Antimicrob Agents Chemother.* 22:499-506; Livingstone et al. 1995, *J. Infect Dis.* 172:1238-45), most methods for addressing this problem mainly use equilibrium dialysis, ultracentrifugation and ultrafiltration. To our knowledge, the binding affinities of PIs with AAG have not been determined using calorimetric methods. Moreover, the measurement of the thermodynamic parameters of a binding process provide a more realistic model.

In view of the clinical significance and the medical need to pharmacokinetically characterize protease inhibitors, a convenient and reliable method to measure the equilibrium dissociation constant, or a functional equivalent thereof, for the binding of a particular drug and plasma protein was designed. With the knowledge of the equilibrium dissociation constant, the *in vivo* activity of a particular PI in the presence of plasma proteins can be estimated using the EC50s from *in vitro* assays without plasma proteins. This will allow as well for an improved preclinical evaluation and selection of new PIs for future clinical development. Thus, the present invention concerns a method for determining the binding affinity of protease inhibitors to plasma proteins that is direct, has high sensitivity and is easy to perform using routine laboratory procedures.

[0007] The present invention provides a method that can quantitatively calculate free drug concentrations of protease inhibitors in human plasma, as well as a method to calculate therapeutic amounts and dosage regimens.

[0008] Furthermore, the present invention provides a method that can calculate the effect of plasma proteins on the antiviral activity (EC50 values) of protease inhibitors from their binding affinities to plasma proteins. The present invention provides as well a method that can evaluate the *in vivo* anti-HIV efficacy of PIs in human plasma.

[0009] Often there may be competition between drugs in plasma protein binding, in which agents that are bound tightly, such as coumarin anticoagulants, macrolide or lincosamide antibiotics that bind tightly to alpha-1-acid Glycoprotein (AAG), are able to displace less tightly bound compounds from their binding sites and thus can increase the free form of the drug and improve the biological efficacy (Sommadosi, et al., 1998 US Patent 5,750,493). Therefore, the present invention provides as well a method for selecting compounds that competitively bind with plasma proteins, said selection being useful for co-administering agents to compete for plasma protein binding, so that an increase of the free plasma concentration of protease inhibitors is achieved.

#### Brief description of the drawings

[0010]

Figure 1 shows the Isothermal Titration Calorimetry measurements for the binding of Amprenavir to AAG

Figure 2 shows the Isothermal Titration Calorimetry measurements for the binding of Indinavir to AAG

Figure 3 shows a comparison for various PIs of the calculated EC50, and the experimental EC50 in the presence of 1 mg/mL AAG and in the absence of AAG.

Figure 4 shows the correlation analysis of calculated EC<sub>50</sub> versus experimental EC<sub>50</sub> for various PIs

Figure 5 shows the *in vivo* efficacies of various PIs calculated from their free drug concentrations

Table 1 lists the thermodynamic parameters of various PIs binding to AAG at 37°C in 20mM phosphate buffer, pH 7.4.

Table 2 lists the binding affinities of 3 different PIs to AAG, their free drug concentrations, calculated IC<sub>50</sub>s, calculated efficacy, and IC<sub>50</sub>s values obtained from antiviral assays in the absence of AAG.

## Detailed description

[0011] At all drug concentrations, the amount of a protein-bound drug depends on the affinity constant and the protein concentration and the present invention concerns a method to calculate the concentration of free drug available to block viral replication from the protein-drug affinity constants under physiologic circumstances. High sensitivity isothermal titration calorimetry (ITC) has been used in the present method to directly measure the binding thermodynamics, such as binding affinities ( $K_a$ ), enthalpies ( $\Delta H^0$ ), entropies ( $\Delta S^0$ ) and binding free energies ( $\Delta G^0$ ), of various PIs to AAG. Combined with the antiviral EC<sub>50</sub> values, the present method allows to estimate the antiviral EC<sub>50</sub> values in the presence of AAG and the *in vivo* efficacy of PIs. Thus, the present invention provides a method for determining the binding affinity of at least one HIV protease inhibitor to at least one plasma protein comprising: i) providing at least one plasma protein; ii) providing at least one protease inhibitor; and iii) quantifying the binding affinity between the at least one plasma protein and the at least one protease inhibitor based on isothermal titration calorimetry measurement.

[0012] The term binding refers to an interaction or association between a minimum of two entities, or molecular structures, such as a ligand and an antiligand. The interaction may occur when the two molecular structures are in direct or indirect physical contact or when the two structures are physically separated but electromagnetically coupled there between, e.g. by hydrogen bonds or Van der Waals interactions. Examples of binding events of interest in a medical context include, but are not limited to, ligand/receptor, antigen/antibody, enzyme/substrate, enzyme/inhibitor, protein/protein, DNA/DNA, DNA/RNA, RNA/RNA, nucleic acid mismatches, complementary nucleic acids, nucleic acid/proteins, plasma proteins/drugs.

[0013] The binding affinity of two molecules would therefore be the degree of interaction between two molecules. The binding affinity of plasma protein and a protease inhibitor can be expressed using various parameters such as the heat capacity change evolved on association of a protease inhibitor with a plasma protein ( $\Delta C_p$ ); the equilibrium association binding constant ( $K_a$ ) or the equilibrium dissociation constant ( $K_d$ ), the free energy ( $\Delta G$ ), the entropy of binding ( $\Delta S$ ), the enthalpy ( $\Delta H$ ).

[0014] The data analysis of ITC results is performed as described (Freire, et al., 1990, Anal. Chem., Vol 62: 950A-959A). Briefly, the isothermal titration calorimetry (ITC) measurements are designed to obtain primarily the enthalpy of each complex formation and their stoichiometries. The heats of each reaction are determined by integration of the peaks observed. After the contribution from the heat of dilution of each injection is subtracted, the heat is plotted against the molar ratio of PIs to AAG. The binding constant ( $K_a$ ), enthalpy of binding ( $\Delta H^0$ ), and stoichiometry (N) of the formation of complex are determined by fitting the binding isotherm against the binding equation using an independent binding model. Data analysis may be carried out with, for example, MicroCal ORIGIN software, usually provided with the instrument.

[0015] HIV protease inhibitors include those compounds whose mechanism of action comprises an inhibition of the viral protease enzyme. As example, and with no limitation to future new compounds, HIV protease inhibitors include ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), amprenavir (APV), telinavir (SC-52151), tipranavir (TPV), saquinavir (SQV), lopinavir (LPV), atazanavir, palinavir, mozenavir, BMS 186316, DPC 681, DPC 684, AG1776, GS3333, KNI-413, KNI-272, L754394, L756425, LG-71350, PD161374, PD173606, PD177298, PD178390, PD178392, PNU 140135, maslinic acid, U-140690, their prodrugs, metabolites, N-oxides and salts.

[0016] Plasma proteins include all proteins found endogenously in plasma. Examples of plasma proteins include without limitation Albumin (HSA), Alpha-1-acid Glycoprotein (AAG), Alpha-1-Antichymotrypsin, Alpha-1 Antitrypsin AT, alpha-fetoprotein, Alpha-1-microglobulin A1M, Alpha-2-Macroglobulin A2M, Angiostatin, Beta-2-Glyco-protein 1, Beta-2-microglobulin, Beta-2-Microglobulin B2M, Beta-N-Acetyl-glucosaminidase B-NAG, recombinant Centromere Protein B, Collagens (type 1-VI), Complement C1q, Complement C3, Complement C4, Ceruplasmin, Chorionic Gonadotrophin HCG, Chorionic Gonadotrophin Beta CORE BchCG, C-Reactive Protein CRP, CK-MB (Creatine Kinase-MB), CK-MM & CK-BB, Cystatin C, D-Dimer, dsDNA, Ferritins, Glycogen Phosphorylase ISO BB, Haptoglobulin, IgA, IgE, IgG, IgG, IgM, Kappa light chain, lambda light chain, recombinant LKM Antigen, La/SS-B, Lysozyme, Myelin Basic Protein, Myoglobin, Neuron-Specific Enolase, Placental Lactogen, Prealbumin, Pregnancy assoc Plasma Protein A, Pregnancy specific beta 1 glycoprotein (SP1), Prostate Specific Antigen PSA, PSA-A1-Act complex, Prostatic Acid Phosphatase

PAP, Proteinase 3 (PR3/Anca), Prothrombin, Retinol Binding Globulin RBP, recombinant human RO/SS-A 52kda, recombinant human RO SS-A 60kda, Sex Hormone Binding Globulin SHBG, S100 (BB/AB), S100 BB homodimer, Thyroglobulin Tg, Thyroid Microsomal Antigen, recombinant thyroid peroxidase TPO, Thyroid Peroxidase TPO, Thyroxine Binding Globulin TBG, Transferrin, Transferrin receptor, Troponin I complex, Troponin C, Troponin I, Troponin T, Urine Protein 1. Plasma proteins most associated with protease inhibitors include albumin,  $\alpha_1$ -acid glycoprotein and lipoproteins.

**[0017]** Plasma proteins may also encompass proteins of external origin, which are not necessarily forming part of the physiological population, but may be found in the body, i.e. proteins from diet origin or from drug compositions.

**[0018]** Particular plasma proteins may have several variants. The term protein variant refers to a polypeptide comprising one or more substitutions of the specified amino acid residues underlying the protein. The total number of such substitutions is typically not more than 10, e.g. one, two, three, four, five or six of said substitutions. In addition, the protein variant may optionally include other modifications of the parent enzyme, typically not more than 10, e.g. not more than 5 such modifications. The variant generally has a homology with the parent enzyme of at least 80 %, e.g. at least 85 %, typically at least 90 % or at least 95 %. Variants may not only differ in primary structure (amino acid sequence), but also in secondary or tertiary structure and the amount and structure of covalently attached carbohydrates. A protein may be present in plasma in different variants, at similar or different concentrations. Variants of a protein may exhibit different binding properties. For instance human  $\alpha_1$  acid glycoprotein is present in two different variants, A and F1/S, which have different binding properties to various ligands and drugs.

**[0019]** The methods of the present invention may additionally comprise as part of the test composition, any compound, including, but not limited to, dipeptides, tripeptides, polypeptides, proteins, small and large organic molecules, buffers, or test aid components, and derivatives thereof. In a particular embodiment, the method preferably includes a competitive binding agent. A competitive binding agent refers to those molecules that competitively bind to plasma proteins in the presence of protease inhibitors. Said competitive binding agent could be one or two more drugs, preferably other drugs than antivirals which bind to plasma proteins, also preferably one or two more drugs effective to treat AIDS and related syndromes, more preferably one or two more antivirals, such as NNRTI, NRTI, PI, fusion inhibitors, entry inhibitors, integrase inhibitors, so concomitant administration of antiretrovirals, optionally with other types of drugs, and its influence on plasma protein binding properties may be studied. Therefore, the present invention also provides a method for selecting compounds that bind competitively to plasma proteins in the presence of protease inhibitors. Said compounds may be used as co-administered agents to increase the free plasma concentration of protease inhibitors.

**[0020]** In another embodiment, the different methods of the present invention may comprise physiological fluid, or have the same components thereof, preferably the physiological fluid is blood, more preferably plasma, also preferred is serum.

**[0021]** In another embodiment of the present invention, there is provided a method for determining the concentration of at least one protease inhibitor present in free form within a test medium, said test medium also containing a bound form of said protease inhibitor with at least one plasma protein, in equilibrium with said free form, the method comprising the steps of: i) providing at least one plasma protein, ii) providing at least one protease inhibitor; iii) quantifying the concentration of protease inhibitor in free form based on isothermal titration calorimetry measurements.

**[0022]** Particularly, the quantification of the concentration of protease inhibitor in free form may be performed by direct measurement of binding affinity  $K_d$  using isothermal titration calorimetry; and calculating the concentration of protease inhibitor in free form using the formula:

$$[drug]_{free} = \frac{1}{2} \left[ \sqrt{(P_t - L_t + K_d)^2 + 4K_d L_t} - (P_t - L_t + K_d) \right] \quad \text{Equation 1}$$

where  $P_t$  and  $L_t$  are total protein and drug concentrations, and  $K_d$  is the equilibrium dissociation constant obtained in step (iii).

**[0023]** Optionally, the formula to be applied may be:

$$PL = \frac{(K_a L_0 + nK_a P_0 + 1) - \sqrt{(K_a L_0 + nK_a P_0 + 1)^2 - 4nK_a^2 P_0 L_0}}{2nK_a} \quad \text{Equation 2}$$

where  $PL$  is the protein-bound concentration of PIs,  $L_0$  and  $P_0$  are the total concentration of PIs and plasma protein,  $K_a$  and  $n$  are the binding constants and number of binding sites, respectively. The concentrations of free PIs may be then calculated by subtraction of the bound concentration from the total concentration, equation 3,

$$L_{free} = L_0 - PL$$

Equation 3

**[0024]** Additionally, the method for calculating the free concentration of protease inhibitors available to inhibit viral replication may be performed by obtaining the  $K_a$  (or association constant) from isothermal titration calorimetry measurement under different plasma protein concentrations, preferably physiological concentrations.

**[0025]** The concentration or amount of protease inhibitor present in free form is particularly useful to establish pharmacokinetic characteristics such as the distribution volume, half-life, bioavailability, and to further determine the therapeutic amount, dosage amounts and dosage intervals, necessary to accomplish an effective therapeutic treatment. Thus, the concentration or amount of protease inhibitor present in free form allows the design of a dosage regimen for a given drug. Same information may be used for patient management, therapeutic drug monitoring, thus, to adjust and tailor the dosage regimen for individual patients and conditions.

**[0026]** The present invention further provides a method of constructing a binding affinity and pharmacokinetic profile database of HIV protease inhibitors, with plasma proteins, and variants thereof, comprising: i) providing at least one plasma protein, and variants thereof; ii) providing at least one protease inhibitor; iii) quantifying the binding affinity between the at least one plasma protein and the at least one protease inhibitor based on isothermal titration calorimetry measurements; and/or quantifying the concentration of protease inhibitor in free form based on isothermal titration calorimetry measurements; iv) correlating in a data table the binding affinity or the concentration of protease inhibitor in free form, with the dosage regimens. Said method for constructing such database also encompasses reports that are generated comprising a listing, analysis, or other information regarding the binding affinities, pharmacokinetic parameters, and their correlation to drug dosage regimens identified by the methods of the invention.

**[0027]** In another embodiment of the present invention, there is provided a method for determining the antiviral activity or EC50 value of at least one HIV protease inhibitor in the presence of at least one plasma protein comprising: providing at least one plasma protein; providing at least one protease inhibitor; quantifying the binding affinity between the at least one plasma protein and the at least one protease inhibitor based on isothermal titration calorimetry measurements.

**[0028]** Particularly, the quantification of the antiviral activity or EC50 value of a protease inhibitor in the presence of at least one plasma protein may be performed by direct measurement of binding affinity  $K_a$  using isothermal titration calorimetry; and calculating the antiviral activity or EC50 value of the protease inhibitor using the formula:

$$EC50_{inplasma\ protein} = EC50 + \frac{K_a \cdot C_{plasma\ protein}}{K_a + \frac{1}{EC50}} \quad \text{Equation 4}$$

where,  $EC_{50}$  is the 50% of effective concentration of at least one protease inhibitor in a cell-based assay without plasma proteins, and  $C_{plasma\ protein}$  is the plasma protein concentration.

**[0029]** In another embodiment of the present invention there is provided a method for determining the in vivo efficacy of at least one HIV protease inhibitor in human plasma protein based on determining the concentration of at least one protease inhibitor present in free form as explained above.

**[0030]** Particularly, the quantification of the in vivo efficacy of a protease inhibitor in human plasma may be performed by direct measurement of binding affinity  $K_a$  using isothermal titration calorimetry; calculating the concentration of protease inhibitor in free form using the formulas described above; and calculating the in vivo efficacy of the protease inhibitor in human plasma using the formula:

$$Efficacy = \frac{C_{free}}{EC_{50}} \quad \text{Equation 5}$$

where,  $C_{free}$  is the concentration of protease inhibitor in free form, the  $EC_{50}$  is the 50% of effective concentration of at least one protease inhibitor in a cell-based assay without plasma proteins.

**[0031]** Furthermore, the present invention provides a method of constructing a binding affinity and antiviral activity and/or efficacy database of HIV protease inhibitors, with plasma proteins, and variants thereof, comprising: i) providing at least one plasma protein, and variants thereof; ii) providing at least one protease inhibitor; iii) quantifying the binding affinity between the at least one plasma protein and the at least one protease inhibitor based on isothermal titration calorimetry measurements; and/or quantifying the concentration of protease inhibitor in free form based on isothermal titration calorimetry measurements; iv) correlating in a data table the binding affinity and/or the concentration of protease inhibitor in free form, with the antiviral activity and/or efficacy. Said method for constructing such database also en-

compasses reports that are generated comprising a listing, analysis, or other information regarding the binding affinities, free drug concentrations, pharmacodynamics' parameters, and their correlation to antiviral activities and efficacies determined by the methods of the invention.

[0032] Another embodiment of the invention is a kit comprising i) at least one plasma protein, and ii) at least one protease inhibitor. Said kit may be directed to determining the binding affinity of at least one HIV protease inhibitor to at least one plasma protein. Additionally, the kit may be used for determining the concentration of at least one protease inhibitor present in free form within a test medium, said test medium also containing a bound form of said protease inhibitor with at least one plasma protein in equilibrium with said free form.

[0033] In a similar embodiment of the invention there is a kit comprising at least one plasma protein, and at least one protease inhibitor; and directed to determining the EC<sub>50</sub> value of at least one HIV protease inhibitor to at least one plasma protein. Optionally, the same kit may be used for determining the in vivo efficacy of at least one HIV protease inhibitor in human plasma.

[0034] The kits may further comprise any reagents necessary to practice the methods of the invention and any equipment or apparatus needed to practice the methods of the invention, such as the equipment necessary to measure binding affinity.

[0035] The methods provided in the present invention may optionally be used as or comprise part of a high-throughput screening assay where numerous test compositions are evaluated for their effect on binding affinity of the at least one plasma protein and the at least one protease inhibitor and for the pharmacokinetic derived properties of the protease inhibitors.

[0036] The order of the steps of the methods of the invention may be varied. One of skill in the art would be able to determine which variations in the order of the steps are applicable.

[0037] Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the claims.

#### Example 1: Thermodynamic Equilibrium Binding Assay Using ITC

[0038] Binding experiments were run using Isothermal Titration Calorimetry (ITC) to measure bound and unbound protease inhibitors.

Sample preparation: Solutions of AAG and its variants for ITC experiments were made up in 20mM sodium borate buffer, pH7.4. All compounds were prepared as stock solution of 20mM or 25mM in DMSO and thereafter diluted to desired concentration in the same buffer. For all ITC experiments, the percentage of DMSO is below 1%.

ITC Experiments: The isothermal titration measurements of the binding of various PIs to AAG can be typically carried out at 37°C using a VP-ITC titration calorimeter (MicroCal, Northampton, MA). The instrument was electrically calibrated by means of a standard electric pulse as recommended by the manufacturer. For the PIs binding to AAG, solutions of PI (80~100μM) were used to titrate AAG (10~12μM). A 300μL syringe was used for the titrant, mixing was effected by stirring this syringe at 300 rpm during equilibration and experiment. Typically after a preliminary 2μL injection, 25 injections of 10μL each were performed with a 250s interval between injections in a single titration. The reference cell of the calorimeter filled with water, acts as a thermal reference to the sample cell. To correct for PIs heats of dilution, the control experiments were also performed using similar conditions with buffer solution only.

All solutions were degassed before titrations to reduce the noise. The buffer contained 20mM sodium phosphate, pH 7.4.

[0039] Two typical titration calorimetry measurements under same condition are shown in Figure 1 and 2. The upper panels in Figure 1 and 2 show the traces recorded for each 10μL injection of 80μM APV and IDV into 12μM, 1.45mL of AAG, respectively. The titration profiles for APV and IDV clearly indicate that APV binds to AAG accompanied with an exothermic heat effect, whereas IDV does not bind to AAG under the conditions studied. The area of each peak was integrated and corrected for the PI heat of dilution, which was estimated by a separate experiment by injecting the PI into the buffer. By fitting the titration curve with a nonlinear least-squares method, the enthalpy change  $\Delta H^0$ , and the binding constant  $K_a$  of PI binding to AAG can be estimated with the assumption of an independent binding site model. Lower panels of Figures 1 and 2 show the best least-squares fit of each integrated heat. The results obtained by this curve fitting using the calorimetric software supplied with the calorimeter for the APV binding to AAG are  $K_a = 1.49 (\pm 0.20) \times 10^6 \text{ M}^{-1}$  and  $\Delta H^0 = -14.2 \pm 0.5 \text{ kcal} \cdot \text{mol}^{-1}$ .

[0040] The complete thermodynamics of various PIs binding to AAG at 37°C in 20mM phosphate buffer, pH 7.4 are summarized in Table 1. The values provided are the average of duplicate experiments. For all the PIs studied binding to AAG, we obtained exothermic enthalpy. The standard free energies ( $\Delta G^0$ ) were obtained from the equation  $\Delta G^0 = -RT \ln K_a$ , in which the  $K_a$  is the binding constant at 37°C. The  $\Delta S^0$  function was calculated from the standard thermodynamic relation  $\Delta G^0 = \Delta H^0 - T\Delta S^0$ . The fold increase EC<sub>50</sub> is the change in experimental EC<sub>50</sub>s obtained from cell-based assays in the absence of AAG and in the presence of 1mg/mL AAG, according to the method described by

Pauwels et al., Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds, J. Virol. Methods, 1988, 20(4), 309-21.

Table 1

PIs	Fold Inc. EC <sub>50</sub>	K <sub>a</sub> (10 <sup>6</sup> M <sup>-1</sup> )	ΔH (kcal/mol)
IDV	1.0	No binding	/
RTV	18.9	1.55±0.49	-2.58±0.16
NFV	22.9	2.35±0.42	-4.26±0.09
APV	23.7	1.49±0.20	-14.22±0.49
SC-52151	29.2	2.38±0.60	-1.33±0.05
SQV	5.6	0.34±0.12	-3.57±0.21
LPV	37.1	5.77±0.72	-5.68±0.07
KN1764	N/A	1.24±0.27	-7.65±0.40

**Example 2: K<sub>a</sub> can be utilized to predict the effect of AAG on EC<sub>50</sub> value in cell-based antiviral assays.**

[0041] In order to determine whether there is a strong correlation between the loss of activity of PIs in presence of AAG and their binding affinities measured directly by ITC, the antiviral EC<sub>50</sub> of these PIs in the presence of 1mg/mL of AAG from their binding constants K<sub>a</sub> with AAG and their EC<sub>50</sub> in the absence of AAG was calculated according to Equation 4. Figure 3 shows the calculated and experimental EC<sub>50</sub> of 7 PIs studied here in the presence of 1mg/mL AAG and in the absence of AAG. As discussed above, the addition of 1mg/mL AAG in the cell culture markedly decreased the EC<sub>50</sub> of PIs except IDV, herein we consider the presence of AAG has no effect on EC<sub>50</sub> of IDV. Comparison of the calculated and experimental EC<sub>50</sub> of these PIs in the presence of AAG revealed that the calculated and experimental values were highly consistent (Figure 3). Figure 4 shows the calculated EC<sub>50</sub> versus experimental EC<sub>50</sub>, the correlation analysis between these values yields a slope of 0.982 with a correlation coefficient of 0.96. This excellent correlation strongly convinced the validation of the present calculation method. Importantly, it was demonstrated that the inhibitory effect of AAG on the *in vitro* activity (EC<sub>50</sub>) of PIs is highly correlated with their binding affinities with AAG.

**Example 3: Comparative evaluation of the *in vivo* antiviral efficacy of PIs in the presence of AAG.**

[0042] The evaluation of the relative *in vivo* efficacy of PIs in human plasma from their EC<sub>50</sub> (Equation 5) is shown in Figure 5. The validation of the correct equation was confirmed using published data for three PIs monotherapy trials.

**Example 4: Data table**

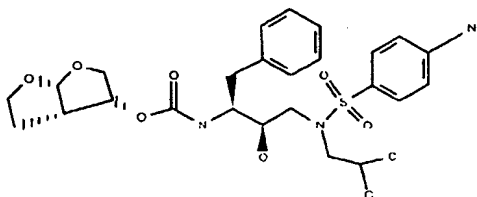
[0043] In table below, a data table with the binding affinity of 3 PI's to AAG (K<sub>a</sub>), their free drug concentrations, calculated IC<sub>50</sub>s, and calculated efficacy were extracted from the ITC results and compared to IC<sub>50</sub>s from an antiviral assay in the absence of AAG. The predicted and experimental IC<sub>50</sub>s showed a good correlation.

Table 2

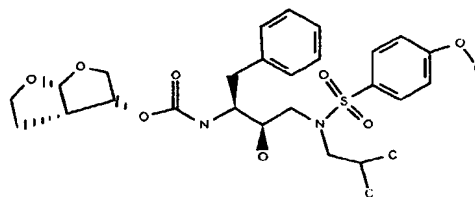
ID	IC <sub>50</sub>	K <sub>a</sub> to AAG	Predicted IC <sub>50</sub> @ 1mg/mL AAG	Free drug Concentration (M)	"Efficacy "
1	3,00E-09	1,92E+06	7,46E-08	1,30E-06	432,16
2	8,00E-10	2,19E+06	2,27E-08	1,19E-06	1484,7
3	5,00E-10	1,24E+06	8,25E-09	1,71E-06	3419,8



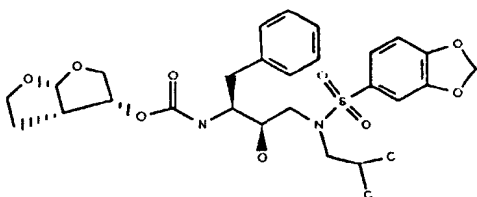
Compound number 1



Compound number 3



Compound number 2



### Claims

1. A method for determining the binding affinity of at least one protease inhibitor to at least one plasma protein comprising:
  - i) providing at least one plasma protein;
  - ii) providing at least one protease inhibitor;
  - iii) quantifying the binding affinity between the at least one plasma protein and the at least one protease inhibitor based on isothermal titration calorimetry measurements.
2. A method of selecting compounds that bind competitively to plasma proteins in the presence of protease inhibitors, said compounds to be used as co-administered agents to increase the free plasma concentration of protease inhibitors, said method based on determining the binding affinity of at least one protease inhibitor to at least one plasma protein according to claim 1.
3. A method for determining the concentration of at least one protease inhibitor present in free form within a test medium, said test medium also containing a bound form of said protease inhibitor with at least one plasma protein in equilibrium with said free form, the method comprising the steps of:
  - i) providing at least one plasma protein;
  - ii) providing at least one protease inhibitor;
  - iii) quantifying the concentration of protease inhibitor in free form based on isothermal titration calorimetry measurements.
4. A method to design a dosage regimen based on determining the concentration of at least one protease inhibitor present in free form according to claim 3.

5. A method of constructing a binding affinity and pharmacokinetic profiling database of protease inhibitors, with plasma proteins, and variants thereof, comprising

5 i) providing at least one plasma protein, and variants thereof;  
 ii) providing at least one protease inhibitor;  
 iii) quantifying the binding affinity between the at least one plasma protein and the at least one protease inhibitor based on isothermal titration calorimetry measurements; and/or quantifying the concentration of protease inhibitor in free form based on isothermal titration calorimetry measurements;  
 10 iv) correlating in a data table the binding affinity or the concentration of protease inhibitor in free form, and dosage regimens.

6. A method for determining the antiviral activity or EC50 value of at least one protease inhibitor in the presence of at least one plasma protein based on determining the binding affinity between the at least one plasma protein and the at least one protease inhibitor according to claim 1.

7. A method for determining the in vivo efficacy of at least one protease inhibitor in human plasma based on determining the concentration of at least one protease inhibitor present in free form according to claim 3.

8. A method of constructing a binding affinity and antiviral activity and/or efficacy database of protease inhibitors, with plasma proteins, and variants thereof, comprising

i) providing at least one plasma protein, and variants thereof;  
 ii) providing at least one protease inhibitor;  
 25 iii) quantifying the binding affinity between the at least one plasma protein and the at least one protease inhibitor based on isothermal titration calorimetry measurements; and/or quantifying the concentration of protease inhibitor in free form based on isothermal titration calorimetry measurements;  
 iv) correlating in a data table the binding affinity or the concentration of protease inhibitor in free form, and antiviral activities or efficacies.

9. A method according to anyone of claims 1 to 8, wherein the plasma protein is chosen from albumin,  $\alpha_1$ -acid glycoprotein, lipoproteins, and variants thereof.

10. A method according to anyone of claims 1 to 8, wherein the method further comprises at least one competitive binding agent.

11. A method according to anyone of claims 1 to 8, wherein the method further comprises one or two more drugs.

12. A kit for determining the binding affinity of at least one protease inhibitor to at least one plasma protein comprising:

40 i) at least one plasma protein, and variants thereof;  
 ii) at least one protease inhibitor.

13. A kit for determining the concentration of at least one protease inhibitor present in free form within a test medium, said test medium also containing a bound form of said protease inhibitor with at least one plasma protein in equilibrium with said free form, the kit comprising:

i) at least one plasma protein, and variants thereof;  
 ii) at least one protease inhibitor.

14. A kit for determining the antiviral activity or EC50 value of at least one protease inhibitor in the presence of at least one plasma protein, the kit comprising:

i) at least one plasma protein, and variants thereof;  
 ii) at least one protease inhibitor.

15. A kit for determining the in vivo efficacy of at least one protease inhibitor in human plasma, the kit comprising:

i) at least one plasma protein, and variants thereof;

**EP 1 265 073 A2**

ii) at least one protease inhibitor.

**16.** A kit according to anyone of claims 12 to 15, wherein the plasma protein is chosen from albumin,  $\alpha_1$ -acid glycoprotein, lipoproteins, and variants thereof.

**17.** A kit according to anyone of claims 12 to 15, wherein the kit further comprises at least one competitive binding agent.

**18.** A kit according to anyone of claims 12 to 15, wherein the kit further comprises one or two more drugs.

**19.** A method according to claims 1 to 11 suitable for high throughput screening.

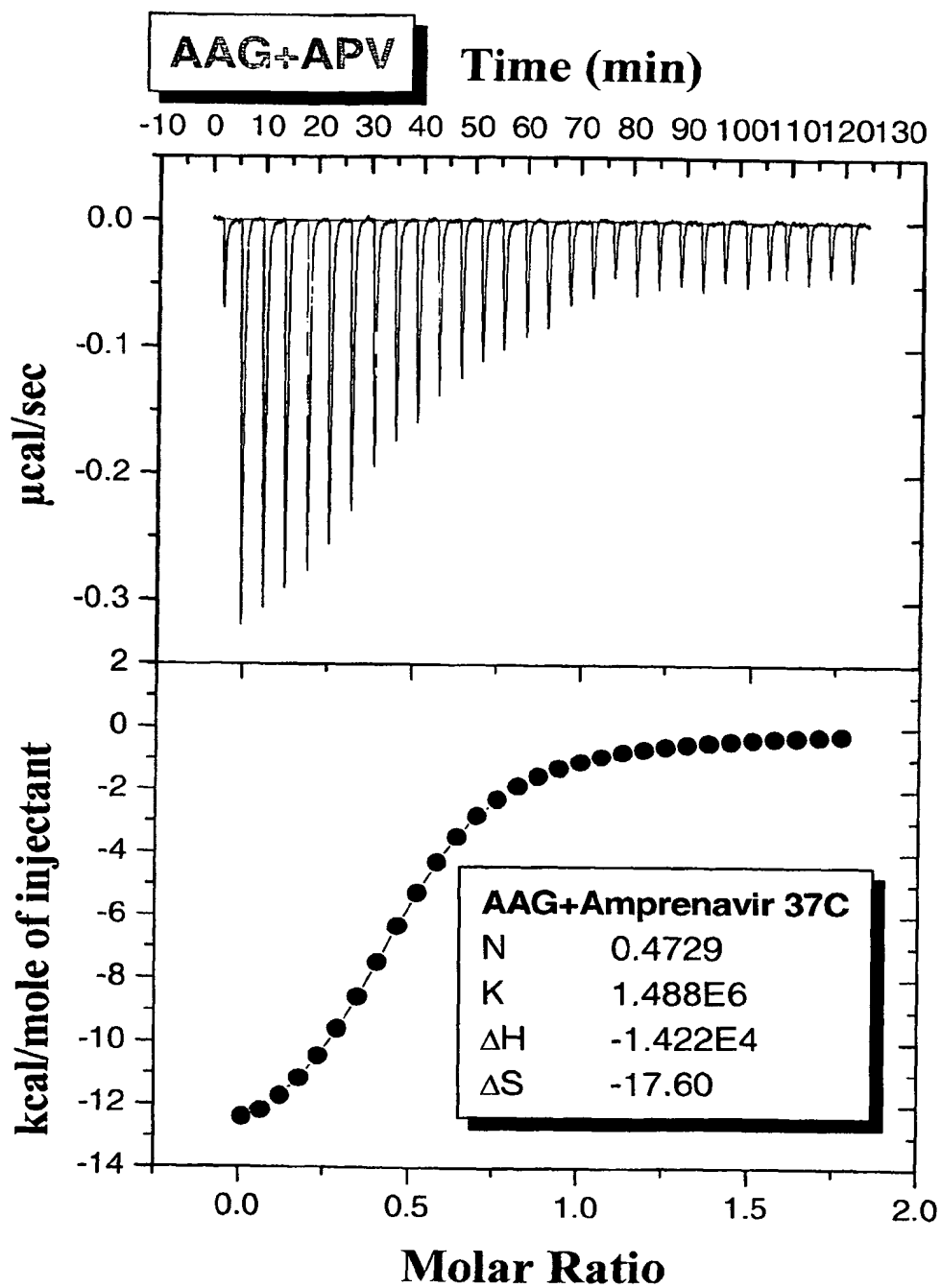


Figure 1

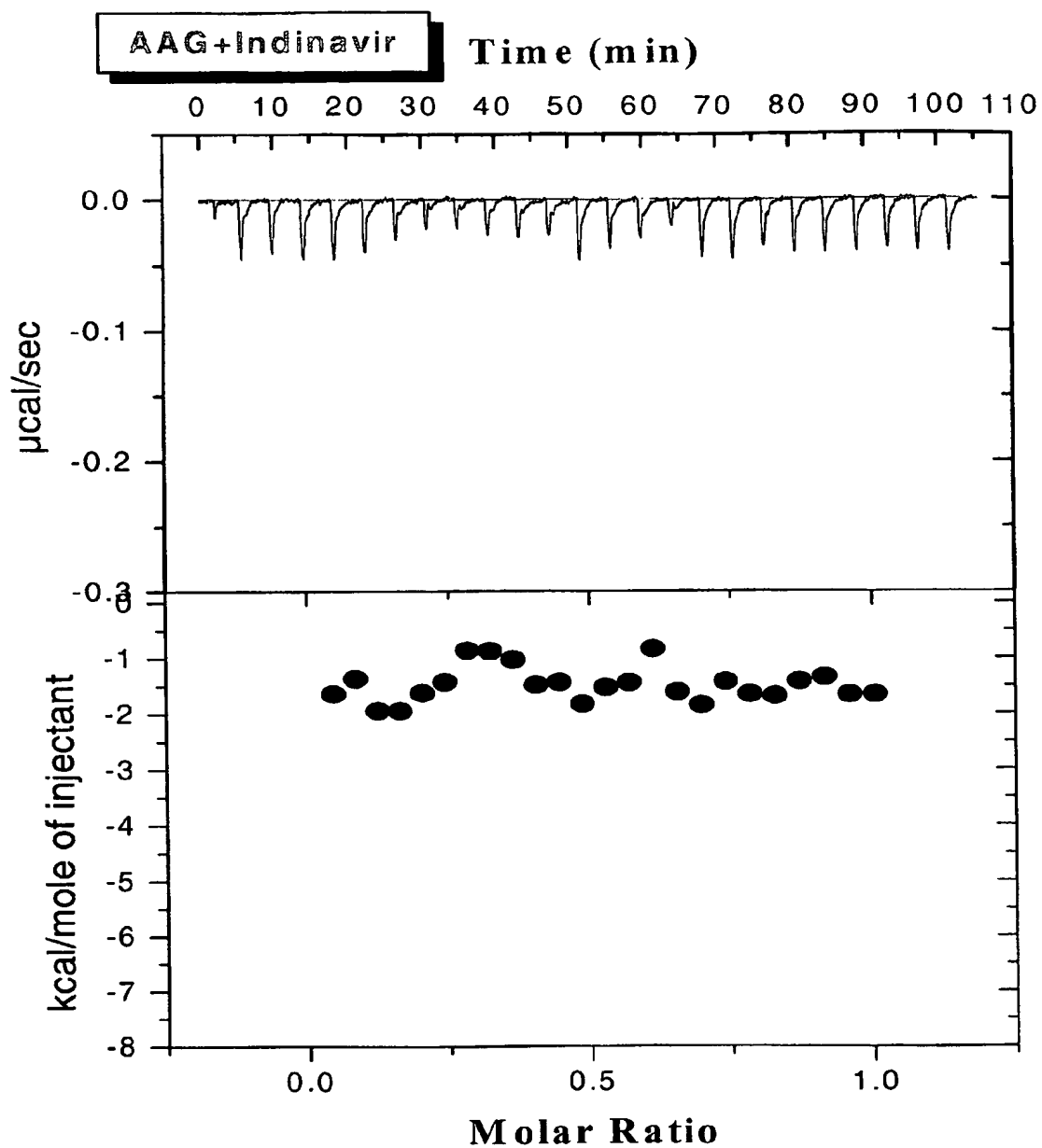


Figure 2

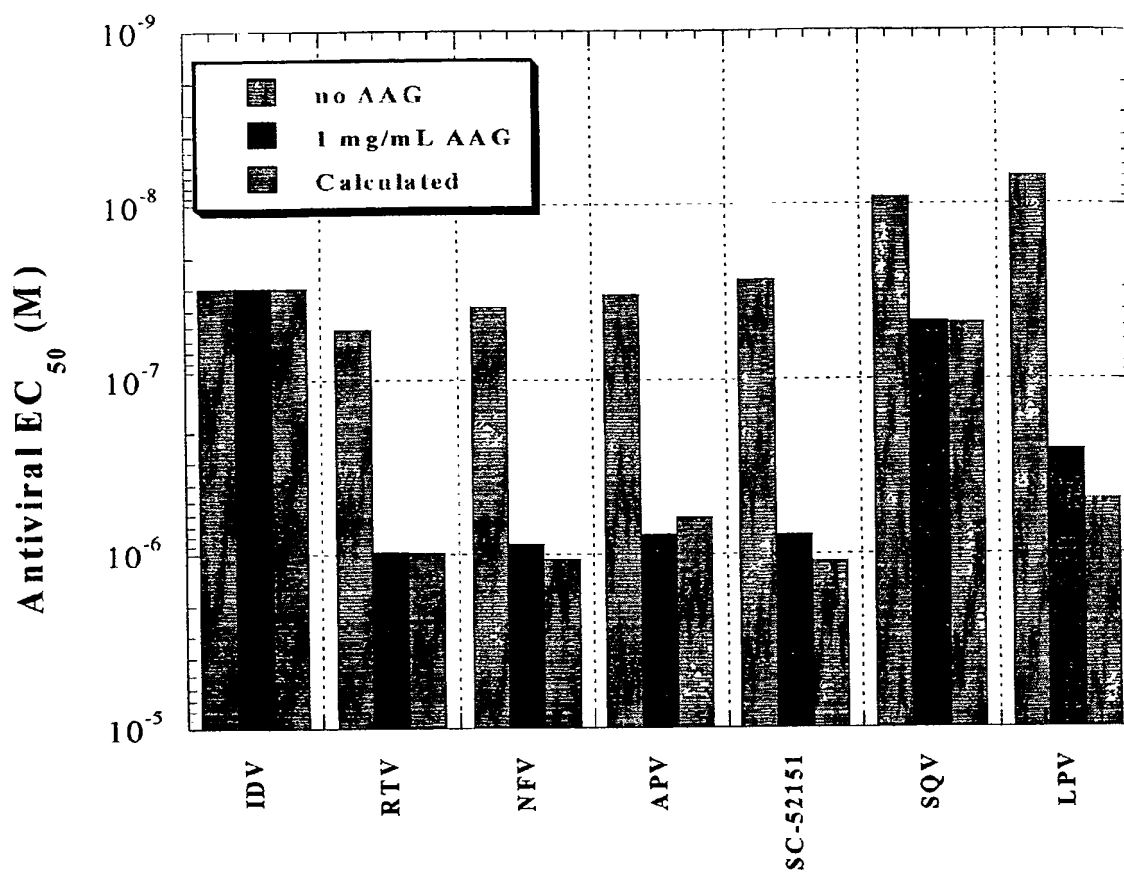


Figure 3

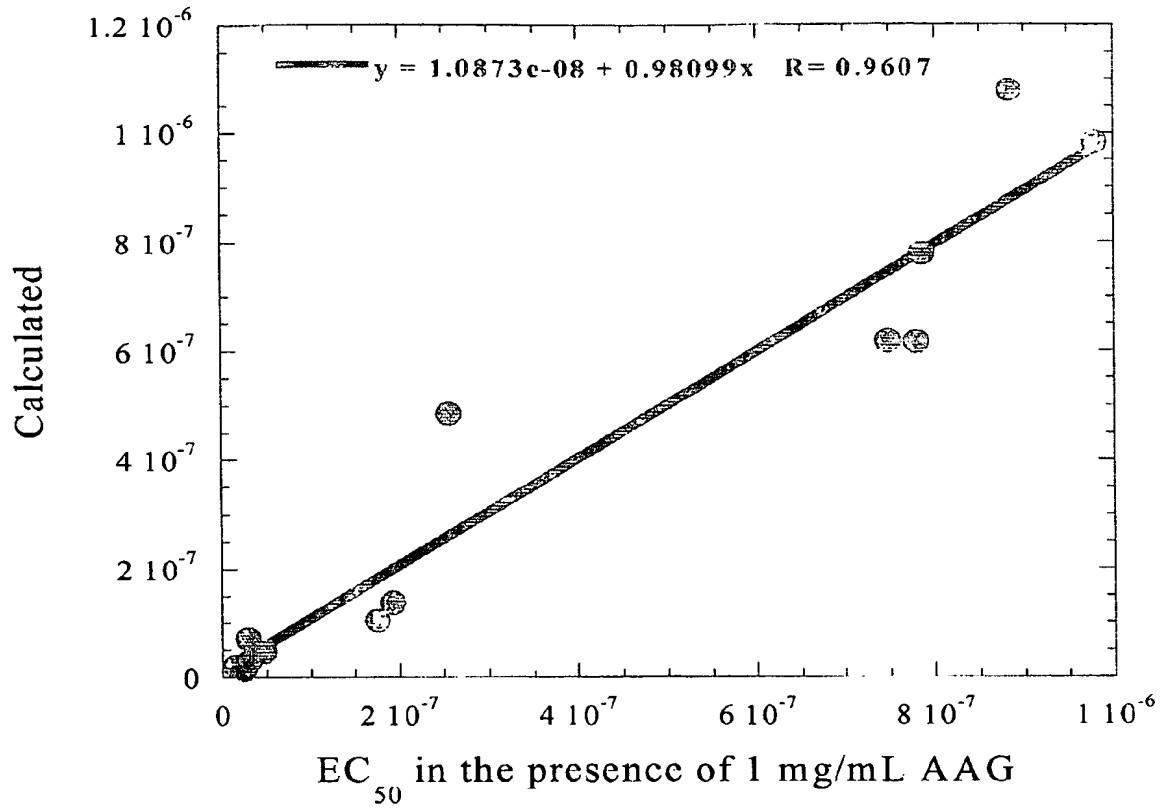


Figure 4

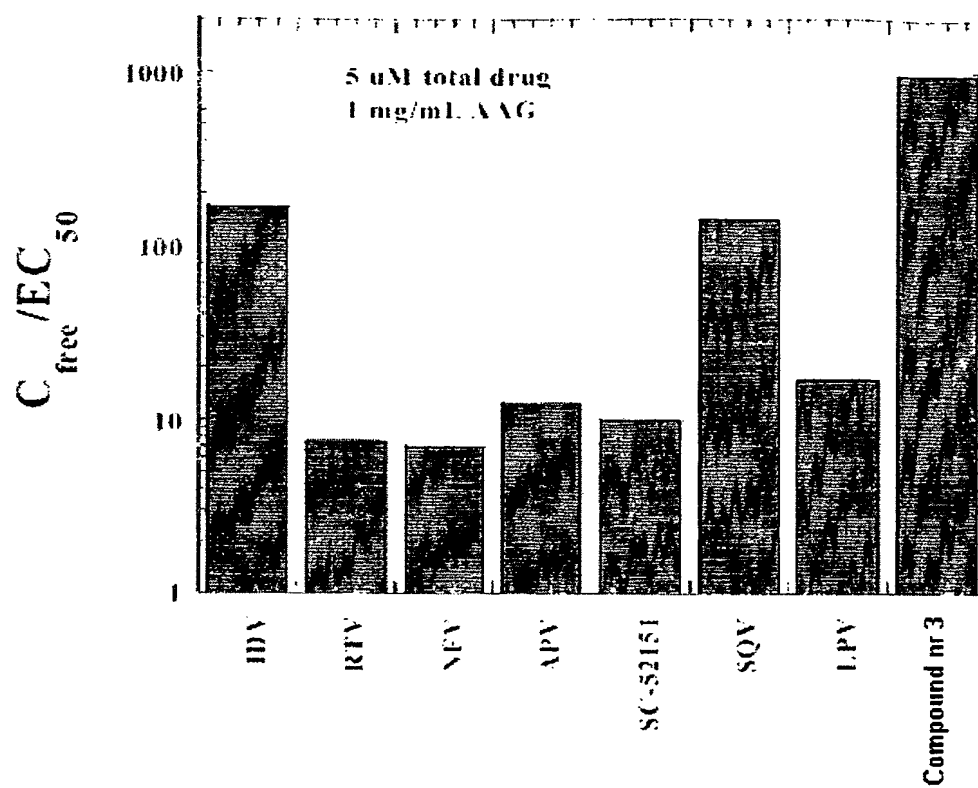


Figure 5



(19)



Europäisches Patentamt  
European Patent Office  
Office européen des brevets



(11)

EP 1 265 073 A3

(12)

## EUROPEAN PATENT APPLICATION

(88) Date of publication A3:  
26.11.2003 Bulletin 2003/48

(51) Int Cl.7: **G01N 33/94**, G01N 33/569,  
G01N 33/68, C12Q 1/37

(43) Date of publication A2:  
11.12.2002 Bulletin 2002/50

(21) Application number: **02077275.2**

(22) Date of filing: **05.06.2002**

(84) Designated Contracting States:  
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU  
MC NL PT SE TR**  
Designated Extension States:  
**AL LT LV MK RO SI**

(72) Inventors:  
• Xie, Dong  
Germantown, Midland 20874 (US)  
• Cao, Wei  
Centereach, New York 11720 (US)  
• Erickson, John W.  
Frederick, Midland 21703 (US)

(30) Priority: **05.06.2001 US 295557 P**

(71) Applicant: **Tibotec Pharmaceuticals Ltd.**  
**Blanchardstown, Dublin 15 (IE)**

(74) Representative: **Daelemans, Frank F.R. et al**  
**Tibotec-Virco N.V.,**  
**General De Wittelaan L11 B3**  
**B-2800 Mechelen (BE)**

(54) **Methods for determining plasma free drug concentration**

(57) The present invention relates to methods for isothermal titration calorimetry analysis of the binding affinity of protease inhibitors to plasma proteins.

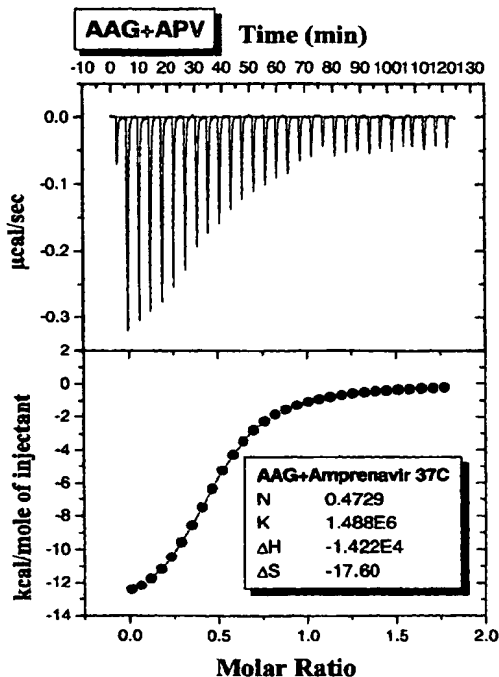


Figure 1



European Patent  
Office

# PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention EP 02 07 7275 shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	JONES K ET AL: "Effect of alphas-acid glycoprotein on the intracellular accumulation of the HIV protease inhibitors saquinavir, zidovudine and zalcitabine in vitro." BRITISH JOURNAL OF CLINICAL PHARMACOLOGY, vol. 51, no. 1, January 2001 (2001-01), pages 99-102, XP002252517 ISSN: 0306-5251 * page 99 * * page 101, left-hand column, paragraph 2 - page 101, right-hand column, paragraph 1 *		G01N33/94 G01N33/569 G01N33/68 C12Q1/37
A	VELAZQUEZ-CAMPOY ADRIAN ET AL: "HIV-1 protease inhibitors: Enthalpic versus entropic optimization of the binding affinity." BIOCHEMISTRY, vol. 39, no. 9, 7 March 2000 (2000-03-07), pages 2201-2207, XP002254944 ISSN: 0006-2960 * page 2201 - page 2203 * ---	1-19	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
			C12Q G01N
<b>INCOMPLETE SEARCH</b> The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims. Claims searched completely :  Claims searched incompletely :  Claims not searched :  Reason for the limitation of the search: see sheet C			
Place of search		Date of completion of the search	Examiner
MUNICH		18 September 2003	Cuendet, P
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

EPO FORM 1503 03 02 (P/C07)



European Patent  
Office

INCOMPLETE SEARCH  
SHEET C

Application Number

EP 02 07 7275

Claim(s) searched incompletely:  
1-19

Reason for the limitation of the search:

Present claims 1-19 relate to an extremely large number of possible methods/kit; this is due to the very broad formulation of the involved products. Support within the meaning of Article 84 EPC and/or disclosure within the meaning of Article 83 EPC is to be found, however, for only a very small proportion of the method/kit as claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those part of the claims which appear to be supported and disclosed, namely those parts relating to HIV protease inhibitor/antiviral EC50 values in the presence of alpha1-acid glycoprotein; see present desc. p.5, lines 16-29/drawings/examples.



European Patent  
Office

## PARTIAL EUROPEAN SEARCH REPORT

Application Number  
EP 02 07 7275

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A	WO 97 08180 A (UNIV ALABAMA ;SCHINAZI RAYMOND F (US)) 6 March 1997 (1997-03-06) * claims 7,21 *	1-19	
P,A	WO 01 40758 A (UNIV JOHNS HOPKINS) 7 June 2001 (2001-06-07) * page 3 *	2,19	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)

EPO FORM 1503 03.02 (P04C10)

**ANNEX TO THE EUROPEAN SEARCH REPORT  
ON EUROPEAN PATENT APPLICATION NO.**

EP 02 07 7275

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

18-09-2003

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9708180 A	06-03-1997	US 5750493 A	12-05-1998
		AU 716821 B2	09-03-2000
		AU 6860196 A	19-03-1997
		EP 0876387 A1	11-11-1998
		JP 2001500471 T	16-01-2001
		WO 9708180 A1	06-03-1997
-----			
WO 0140758 A	07-06-2001	US 6242190 B1	05-06-2001
		AU 3972001 A	12-06-2001
		CA 2393422 A1	07-06-2001
		EP 1234180 A2	28-08-2002
		WO 0140758 A2	07-06-2001
		US 2001008763 A1	19-07-2001
-----			

EPO FORM P0455

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

**THIS PAGE BLANK (USPTO)**